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AN EVALUATION OF SEVERAL METHODS OF PROCESSING PLANTS FOR AUTORADIOGRAPHY

Robert W. Gesink
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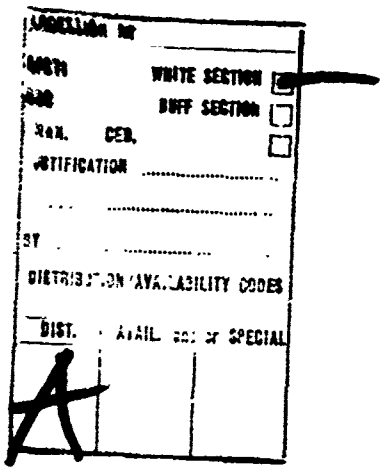

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13. ABSTRACT

Because of the possible errors in interpretation as a result of the occurrence of pseudoimages formed by improper preparation of plant material for autoradiography, three drying methods were compared: freeze-drying, oven-drying, and air-drying. The test species, bean plants and white ash seedlings, were grown in nutrient solution in a growth chamber at about 25 C under 1,400 ft-c of light. Carboxyl-C^{14}-labeled picloram, a representative auxin-type herbicide, was foliarly applied in 10-µl droplets; each plant received 24 µg (0.1 µc) in 100 µl solution containing a surfactant. Bean and ash plants were harvested 5 and 9 days after treatment, respectively. Temperature and time for oven-drying were 95 C for 1 hour, followed by 2 days at 55 C; for air-drying, 25 C at 50% relative humidity for 10 days.

Freeze-drying was the best method for preparing plants for autoradiography, oven-drying the least effective method. Pseudoimages were consistently produced by oven-drying, especially in bean plants; these false images were particularly characterized by peripheral movement of the label in the treated leaves. Sectioning did not prevent this movement. Air-drying was ranked second as an effective technique. Pseudoimages produced by air-dried plants appeared to be relatively minor compared with those of oven-dried plants. The apparent presence of the label in the roots appeared to be the principal anomaly in air-dried bean

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14 KEY WORDS	LINK A		LINK B		LINK C	
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Because of the possible errors in interpretation as a result of the occurrence of pseudoimages formed by improper preparation of plant material for autoradiography, three drying methods were compared: freeze-drying, oven-drying, and air-drying. The test species, bean plants and white ash seedlings, were grown in nutrient solution in a growth chamber at about 25 C under 1,400 ft-c of light. Carboxyl-C^{14}-labeled picloram, a representative auxin-type herbicide, was foliarly applied in 10-µl droplets; each plant received 24 µg (0.1 µc) in 100 µl solution containing a surfactant. Bean and ash plants were harvested 5 and 9 days after treatment, respectively. Temperature and time for oven-drying were 95 C for 1 hour, followed by 2 days at 55 C; for air-drying, 25 C at 50% relative humidity for 10 days.

Freeze-drying was the best method for preparing plants for autoradiography, oven-drying the least effective method. Pseudoimages were consistently produced by oven-drying, especially in bean plants; these false images were particularly characterized by peripheral movement of the label in the treated leaves. Sectioning did not prevent this movement. Air-drying was ranked second as an effective technique. Pseudoimages produced by air-dried plants appeared to be relatively minor compared with those of oven-dried plants. The apparent presence of the label in the roots appeared to be the principal anomaly in air-dried bean plants. Freeze-drying was not as essential for the woody plants as for the bean plants. Air-dried ash produced excellent autoradiographs similar to those of freeze-dried ash. However, metabolic degradation of the labeled compound may occur during air-drying, resulting in probable loss of the one-for-one relationship between the compound under study and the radioactive tracer.

CONTENTS

FIGURES

I. INTRODUCTION*

Autoradiography** is an excellent tool for the study of the distribution and translocation of labeled compounds within plants. Levi[1] indicated that, although many scientists have used autoradiography as a tool in their research, only a few have investigated the possible errors that may result from pseudoimages that may occur for various reasons. Pseudoimages, or artifacts, may result in the researcher's unwittingly drawing erroneous conclusions.

It is of paramount importance that plants be properly dried prior to preparation of the autoradiographs. Pallas and Crafts[2] pointed out that certain methods of handling plants, both before and during autoradiography, may make accurate interpretation of results difficult and that movement of mobile radioactive tracers during plant manipulation may result in false localizations that are not readily identifiable. Millikan[3] and Rice and Rohrbaugh[4] noted that movement of tracers occurred in plants while they were being air-dried or oven-dried.

Pallas and Crafts[2] compared autoradiographs of freeze-dried plants that had been treated with 2,4-D (carboxyl-labeled) with the autoradiographs of similarly treated plants that had been air-dried between blotters. The comparison revealed that in the air-dried plants there was movement of the label throughout the tissues; in the freeze-dried plants no movement of label occurred. They concluded that this movement occurred during the drying process.

According to Levi,[5] the artifacts reported in much of the literature are probably of a purely physical nature. He suggests that heating renders the cells permeable and allows rather free movement of the tracer in solution from the veins, which dry slowly, to the veinlets and parenchymatous tissue of the leaves, which dry faster.

Nelson and Krotkov[6] stated that in light of the serious nature of artifacts that can be obtained, much of the work in translocation of solutes using autoradiography needs to be repeated.

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** Autoradiography is commonly defined as a process by which an image is produced on a photographic film or plate by the radiations from a radioactive substance in an object that is in close contact with the emulsion. An autoradiograph is the X-ray film on which the image is produced.

The present studies were initiated, therefore, to compare various methods of drying plants for autoradiography with respect to the occurrence of pseudoimages. Picloram (4-amino-3,5,6-trichloropicolinic acid) labeled in the carboxyl group with C^{14} was used in these studies as a representative auxin-type herbicide. Comparisons were made among freeze-dried, oven-dried, and air-dried beans (Phaseolus vulgaris L. var. Black Valentine) and white ash (Fraxinus americana L.). Additionally, an effort was made to identify the cause of certain other types of pseudo-images that have occurred sporadically in tracer work conducted in this laboratory.

II. MATERIALS AND METHODS

A. STUDIES WITH BEANS

Bean plants were germinated in washed silica sand and transferred to 1-liter plastic pots of aerated half-strength Hoagland's nutrient solution. The plants were grown in a controlled environment growth chamber at a temperature of 25±0.5 C and a relative humidity of 50±5%. A 16-hour photoperiod of 1,400 ft-c of illumination was provided at plant-top level by a combination of cool white fluorescent lamps and incandescent bulbs.

Plants were selected for experimental use when the primary leaves were fully developed and the first trifoliolate leaf had just opened (9 days old). The plants were treated by applying 50 µl (0.05 µc/100 µl) of C^{14}-labeled picloram (carboxyl-labeled) per leaf to the upper surface of both primary leaves of each plant. This solution contained 0.2% v/v Tween 20. The herbicidal solution was applied in a semicircle as five 10-µl droplets about 1 cm from the basal end of each leaf. The droplets were placed on each of the five main leaf veins because this position was exactly reproducible from plant to plant.

Five days after treatment the plants were harvested in groups of four and utilized in the various drying methods under consideration.

Four plants were quick-frozen immediately after harvest and then freeze-dried for days at -20 C according to the method described by Crafts and Yamaguchi.[7] The freeze-dryer used (Fig. 1) is similar to the one described by Crafts and Yamaguchi with a few modifications. Four freeze-dry vacuum chambers are placed in a standard household freezer and connected to a two-stage gas ballast pump with high vacuum rubber tubing. Before the rubber tubing from the chambers connects to the pump, it channels the air from the tanks through a dry ice - acetone vapor (H_2O) trap located between the freezer and the pump. A more detailed view of an individual chamber is shown in Figure 2.

FIGURE 1. Four Freeze-Dry Chambers Arranged Within Conventional Freezer.

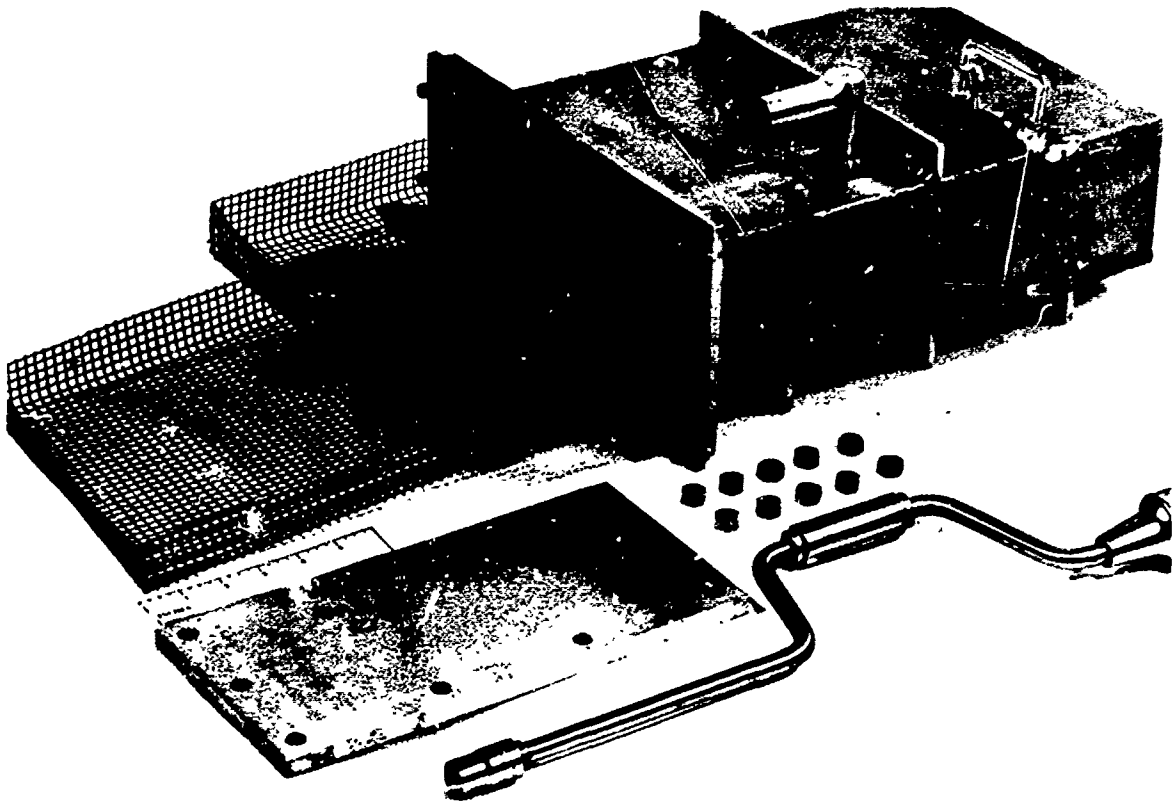

FIGURE 2. Close-Up View of an Individual Freeze-Dry Chamber.

A second group of plants was prepared for oven-drying. Two of the four plants were sectioned as follows: at the root collar, the base of the primary leaf petioles, the stem just above the first node, the base of the first trifoliolate petiole, and at the stem just above the first trifoliolate node. All four plants were then placed between blotters and sheets of cardboard and dried in an oven at 95 C for 1 hour, followed by 2 days at 55 C.

The last four bean plants were placed between blotters, stacked between sheets of cardboard, and air-dried in a growth chamber at a temperature of 25 C and relative humidity of 50%. Autoradiographs were prepared by exposing the freeze-dried plants to Kodak No-Screen X-ray film (Fig. 3) for 31 days; the oven-dried and air-dried plants were exposed for 24 days.

B. STUDIES WITH ASH SEEDLINGS

Four-week-old seedlings of white ash were transferred from vermiculite to 1-liter plastic pots of aerated half-strength Hoagland's nutrient solution and maintained in a controlled-environment growth chamber.

Seedlings were selected for use when they had attained a height of 15 to 20 cm and had five or six whorls of leaves (approximately 5 weeks old). The plants were treated by applying 50 µl (0.10 µc/100 µl) of C^{14}-labeled picloram (carboxyl-labeled) per leaf to the upper surface of the two leaves at the fourth whorl above the root collar. The sublethal dosages of labeled picloram in 0.2% v/v Tween 20 were applied as five 10-µl droplets placed within five lanolin rings on the upper surface of each treated leaf. Greenham and Pook[8] recently reported a new method for applying labeled herbicides to the stems of woody plants in which they were able to eliminate the lanolin step; however, we found no practical method to eliminate this step when making foliar applications.

The plants were harvested 9 days after treatment and dried according to the three methods described in Section II, A. In this study, however, the oven-dried plants were not sectioned. Also the exposure period for preparation of the autoradiographs was 26 days.

C. PSEUDOIMAGES

Four various types of materials were selected to investigate pseudo-images caused by mechanical pressure on the film.

The materials used were aluminum rods, red pine branches (Pinus resinosa Ait.), and stems of beans and ash seedlings. All four materials had about a 0.5-cm diameter and were about 20 cm in length.

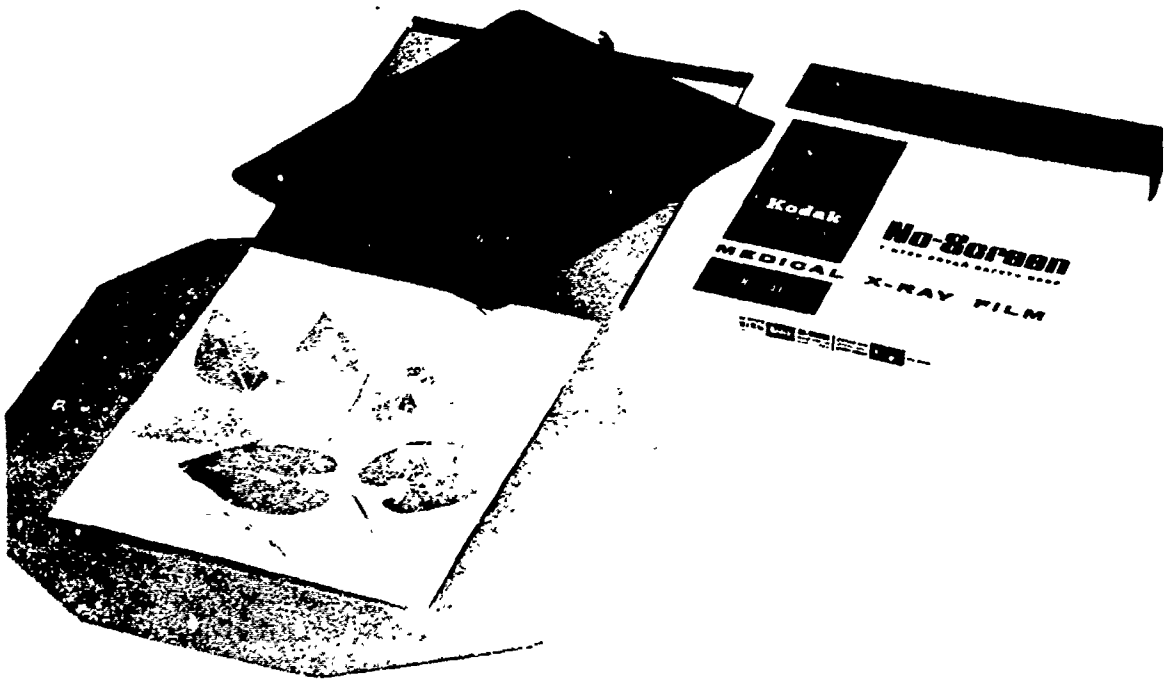

FIGURE 3. Mounted Bean Plant in Film Holder Ready for Exposure to X-Ray Film.

A 3-week exposure was made by placing all four materials on each of three sheets of Kodak No-Screen X-ray film. Thirty-five g/cm^2 of pressure were applied to the materials on the first sheet of film, the materials on the second sheet received 430 g/cm^2, and the third ones received 1,570 g/cm^2. There were three replications of this arrangement. These pressures were maintained for 3 weeks, at which time the film was developed and the materials were again placed on film for a 6-week period.

In addition to investigating pseudoimages due to pressure, an effort was also made to determine whether or not the isotope of potassium (K^{40}) contained in the plant materials was causing a pseudoimage on the X-ray film. Bean plants were grown in half-strength Hoagland's with three different concentrations of potassium. The three rates of application were the normal concentration of potassium in half-strength Hoagland's, 5X normal concentration, and 10X normal concentration. Four plants were grown in each of the different solution cultures for 7 days and then harvested. The leaves and lower one-third of the roots were sectioned from the plants and discarded. The remaining portion of the plants was air-dried and exposed to film for 6 weeks.

III. RESULTS AND DISCUSSION

A. STUDIES WITH BEANS

The plants freeze-dried for 7 days (Fig. 4) show localization of the radioactive material at the points of application, in the stems, and in the apical portions of the plant. Also, there is an absence of an appreciable quantity of the radioactive tracer in the roots. On the basis of past experience in this laboratory and information in the literature, this autoradiograph appears to represent a true picture for the localization of an auxin-type herbicide in a plant at the time of harvest, i.e., when the plant was quick-frozen by pulverized dry ice (about -66 C). Other than past experience, there are a number of criteria available to make the judgment that this is a true representation of where the C^{14} was at time of harvest. One of them is the presence of discrete, circular spots on the treated leaves where the 10-µl droplets were applied. The absence of such well-defined spots is an indication that something went wrong during the lyophilization process. An example of this is presented in Figure 5, which shows a plant that thawed during the freeze-drying process. When a plant thaws before all the water has been removed by sublimation, the cell walls rupture and water films move within the plant, carrying the C^{14}-label into areas that did not actually contain the label at the time of harvest. The drying cycle of the plant in Figure 5 was interrupted 2 days after the plant was quick-frozen because of a vacuum leak in the freeze-dryer, and the label moved into the leaf veins during this interruption. Levi[5] reported similar results; he stated that if the leaves were allowed to thaw at any time during freeze-drying the veins would become heavily labeled.

FIGURE 4. Plant (left) and Autoradiograph (right) of a Treated Bean Plant Freeze-Dried for 7 Days.

FIGURE 5. Plant (left) and Autoradiograph (right) of a Bean Plant that Thawed During Freeze-Drying.

In regard to the number of days required for freeze-drying, it should be noted that in preliminary studies bean plants were adequately dried in 2 days, rather than 7 days, provided that the freeze-dry chambers contained only a very limited number of plants. When the chambers are filled to capacity, however, we found that at least 7 days were necessary to dry the plants.

Three of the autoradiographs produced by the oven-dried plants are shown in Figures 6, 7, and 8. The most characteristic feature of these plants is the peripheral movement of label in the primary leaves. The treatment spots are faint and the label had spread throughout the leaf, indicating a great deal of movement during the drying period. The contrast between the autoradiographs of the oven-dried plants and those of the freeze-dried plants (Fig. 4) is striking.

In regard to the sectioned and nonsectioned plants, an attempt was made to follow and locate the movement of the radioactive tracer during oven-drying. Therefore, two of the four plants were sectioned in order to compare the movement of the tracer in them with its movement in two nonsectioned plants dried in the same manner. An examination of Figures 6, 7, and 8 shows that the label moved into the first trifoliolate leaf of the non-sectioned plants, but not into the trifoliolate leaf of the sectioned plants. Also note the distinct root image in Figure 7 and the absence of a noticeable image in Figure 8. The absence of distinct root image in Figure 6 is somewhat atypical. In our preliminary studies with oven-dried, nonsectioned plants, the radioactive material was always found in both the roots and first trifoliolate leaves.

The autoradiographs of the air-dried plants (Fig. 9) show that some movement of label occurred during the drying period. The movement, however, appears to be only slight compared with that in the oven-dried plants. Also, the label has moved into the roots, but not into the trifoliolate leaf or veins of the primary leaves. The presence of the label in the roots appears to be the principal artifact found in the air-dried plants.

B. STUDIES WITH ASH SEEDLINGS

An autoradiograph of an ash seedling freeze-dried for 7 days is shown in Figure 10. The treatment spots are localized and the label is found primarily in the treated leaves, stems, and growing tips. The autoradiograph is typical of a properly freeze-dried plant and indicates that no movement of materials occurred in the plant during the freeze-drying process.

In contrast to the lyophilized seedlings, the radioactive tracer did move considerably in the oven-dried plants (Fig. 11). The treatment spots spread, and the veins of the treated leaves are heavily labeled. However, when compared with the oven-dried beans, it is evident that the movement of label in the woody plants was less. Also, the roots of the ash seedlings resulted in only a faint image, which is contrary to observations in the oven-dried beans.

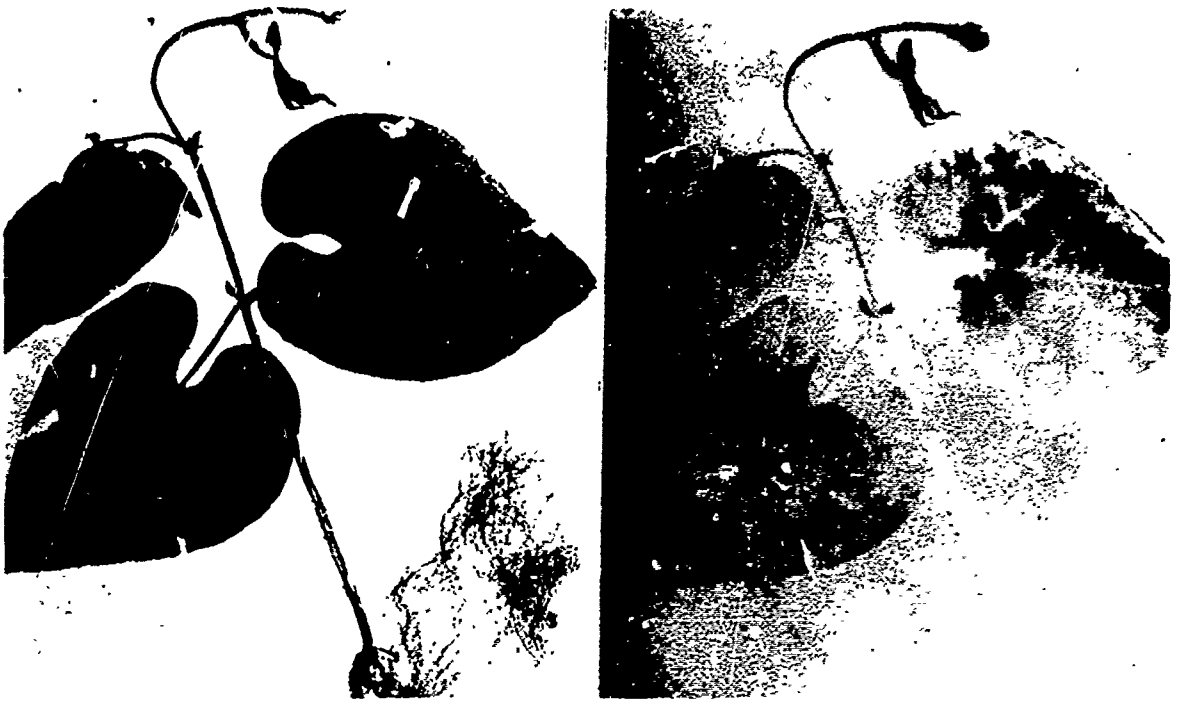

FIGURE 6. Plant (left) and Autoradiograph (right) of a Bean Plant (Nonsectioned) Dried in an Oven at 95 C for 1 Hour Followed by 2 Days at 55 C.

FIGURE 7. Plant (left) and Autoradiograph (right) of a Bean Plant (Nonsectioned) Dried in an Oven at 95 C for 1 Hour Followed by 2 Days at 55 C.

FIGURE 8. Plant (left) and Autoradiograph (right) of a Bean Plant (Sectioned) Dried in an Oven at 95 C for 1 Hour Followed by 2 Days at 55 C.

FIGURE 9. Plant (left) and Autoradiograph (right) of an Air-Dried Bean Plant.

FIGURE 10. Plant (left) and Autoradiograph (right) of an Ash Seedling Freeze-Dried for 7 Days.

FIGURE 11. Plant (left) and Autoradiograph (right) of an Ash Seedling Dried in an Oven at 95 C for 1 Hour Followed by 2 Days at 55 C.

The autoradiographs of the air-dried ash seedlings (Fig. 12) were similar to those produced by the freeze-dried ash. This is quite different from the air-dried beans, in which there was some movement of the labeled materials, especially into the roots.

C. PSEUDOIMAGES

The X-ray film on which the various materials were placed to check for pseudoimages as a result of pressure did not reveal any darkening of the film after the 3-week exposure period. After the 6-week exposure period, however, the film did show very light images for the materials that had been subjected to a high pressure of 1,570 g/cm^2. From earlier work done in our laboratory, there is evidence that at least 9 weeks of exposure are necessary to produce a rather distinct image on the film as a result of pressure. In light of these results, it appears that pseudoimages due to the pressure of the plant materials or pressure placed on the materials during exposure to X-ray film are of little or no significance when exposure periods do not exceed 6 weeks. It has been reported,[1,9] however, that mechanical pressure, bending or abrasion of an emulsion, and certain chemicals can also cause blackening of the film.

The bean plants grown in half-strength Hoagland's solution with various concentrations of potassium gave negative results after being exposed for 6 weeks. All of the plants gave a very light stem image, but there were no differences in the images due to the varied concentrations of potassium in the solution cultures. Therefore, it appears that the isotope of potassium (K^{40}) does not cause an image on the film during the exposure of plant materials.

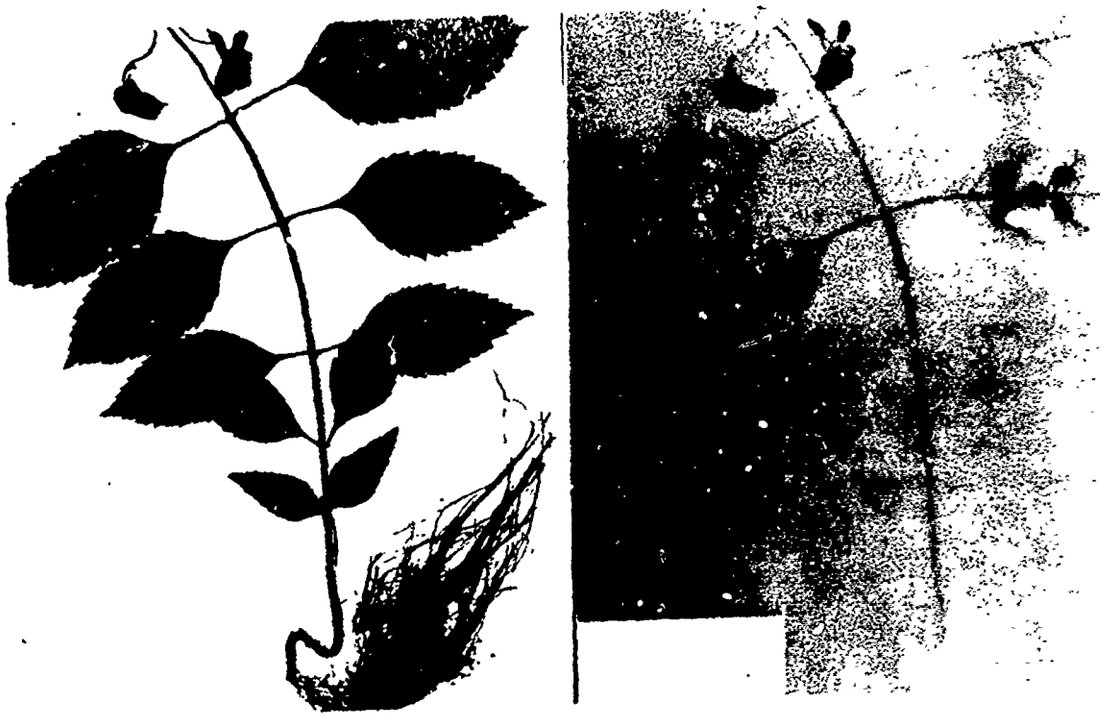

FIGURE 12. Plant (left) and Autoradiograph (right) of an Air-Dried Ash Seedling.

IV. CONCLUSIONS

A comparison of oven- and freeze-dried plants showed that pseudoimages were consistently produced by oven-dried plants, especially bean plants. The pseudoimages produced by these plants were characterized by peripheral movement of the label in the primary leaves. The treatment spots were faint and the label had spread throughout the leaves, indicating that much movement had occurred during the drying process.

Very faint images were produced by the roots and first trifoliolate leaves of the oven-dried plants that had been sectioned prior to drying, but the roots and trifoliolate leaves of the nonsectioned beans produced distinct images. This was further evidence of movement of the tracer in the plant during the oven-drying process.

Pseudoimages were also produced by the air-dried beans but they appeared to be relatively minor compared with those of the oven-dried beans. The presence of the label in the roots appeared to be the major artifact in the air-dried plants.

When the beans and ash seedlings were compared, it was apparent that movement of the tracer was considerably less in the woody plants than in the herbaceous bean plants. This was especially true in the air-dried ash, which produced autoradiographs similar to those of the freeze-dried ash. Thus, air-drying is a more acceptable method for ash than for bean plants.

Our interpretation of artifacts is similar to those of Pallas and Crafts[2] and Levi,[5] who concluded that when plants are killed by quick-freezing or by other similar methods and not maintained in a frozen condition, the cells become permeable. This in turn allows a free movement of solution throughout the plants according to the hydrostatic gradients established during the drying process.

Our results clearly show that freeze-drying is the best method for preparing plants for autoradiography. In our studies, the second most acceptable method of processing plants was air-drying. However, it must be realized that during the air-drying process, metabolic degradation of the labeled compound may occur, with resulting loss of the one-for-one relationship between the compound under study and the radioactive tracer.

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